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Transcription Profiling of the Metal-hyperaccumulator *Thlaspi caerulescens* (J. & C. PRESL)

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Thlaspi caerulescens is a well-studied metal-hyperaccumulator of zinc, cadmium and nickel, belonging to the Brassicaceae family. Moreover it is one of the few hyperaccumulators that occur on different metalliferous soil types, as well as on nonmetalliferous soils. We are interested in the development of systems to improve phytoremediation of metal contaminated soils through improved metal-accumulation. About 1900 cDNAs isolated from *T. caerulescens* roots were hybridized with reverse transcribed RNA from zinc-treated *T. caerulescens* plants of two accessions originating from two different soil types. This comparative transcript profiling of *T. caerulescens* plants resulted in the identification of genes that are affected by heavy metals. The developed microarray proved to be an appropriate tool for a large scale analysis of gene expression in this metal-accumulator species.

Key words: Thlaspi caerulescens, Zinc Hyperaccumulation, Microarray

Introduction

A serious ecological problem all over the world is a long-lasting soil contamination by heavy metals. Metal smelting industry, residues from ore mining, combustion of fossil fuels as well as some pesticides and fertilizers used in agriculture have caused or have at least contributed to this problem. Metal uptake by plants can play a key role in the entry of metals into terrestrial food chains resulting in severe health risks (Vassilev *et al.*, 2004). Therefore it is of great importance to find ways for a remediation of contaminated soils.

Plants can contribute to this challenge with sophisticated concepts. The use of green plants to remove pollutants from the environment or to render them harmless, coined the term "phytoremediation" (Cunningham and Berti, 1993). Depending on the type and area of remediation several methods of phytoremediation are distinguished. A reduction of metal bioavailability by accumulation and precipitation of heavy metals in the root zone is called phytostabilization and removal of heavy metals out of soil by tolerant plants is referred to as phytoextraction (Vassilev *et al.*, 2004). Phytoextraction as a concept is based on metal-hyperaccumulating plants that are able to take up and to tolerate extremely high levels of metals, far beyond those of nonaccumulator plants (Reeves and Baker, 2000).

Thlaspi caerulescens is a well-studied metal-hyperaccumulator of Zn, Cd and Ni, belonging to the Brassicaceae family (Assunção *et al.*, 2003a). Additionally it is one of the few hyperaccumulators that occur on different metalliferous soil types as well as on nonmetalliferous soils (Meerts and Van Isacker, 1997). Healthy *T. caerulescens* plants can grow on metal-rich nutrient solutions containing up to 30,000 μg Zn g^{-1} DW (Brown *et al.*, 1995), 14,000 μg Cd g^{-1} DW (Lombi *et al.*, 2000) or 4,700 μg Ni g^{-1} DW (Schat *et al.*, 2000).

Here we report on the development of a microarray with spotted *T. caerulescens* cDNAs and present the results of comparative transcript profiling of roots from two different *T. caerulescens* accessions grown at different Zn exposures. In addition results on effects of different treatments with zinc on transcriptional profiling in the roots of each accession are shown.

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Materials and Methods

Plant material

Culture of *Thlaspi caerulescens* plants and the climate conditions in the climate chambers were as described by Assunção *et al.*, (2003b). Plant accessions were originally collected at two different sites: a nonmetalliferous soil (Lellingen, Luxembourg) and a soil highly contaminated with Zn, Cd and Pb (La Calamine, Belgium). Seeds of the two origins were grown first on moist peat for three weeks and then plants were transferred to 1-l polyethylene pots (three seedlings per pot) filled with modified half-strength Hoagland's solution. After one week of growth plants were transferred to the same solution, albeit with different Zn concentrations (0, 2, 10, 100 and 1000 μM $ZnSO_4$), for an additional two weeks (Assunção *et al.*, 2003b). During harvest, roots and shoots of each plant were carefully separated, frozen in liquid nitrogen and stored at −70 °C.

Extraction of total RNA

Roots of nine to eleven plants were homogenized in liquid nitrogen. Total RNA was extracted from about 120 mg of the homogenized material with 1 ml of TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions, with minor modifications: both the complete chloroform step for phase separation and the complete RNA wash step were repeated once. RNA concentration was measured using an Ultrospec 3100 pro spectrophotometer (Amersham Biosciences, Freiburg, Germany). For quantitative RT-PCR, remaining genomic DNA was digested with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany). Extracted RNA was checked every time on a 1.5% (w/v) agarose gel prior to and after DNase I digestion.

DNA array

For the *Thlaspi caerulescens* microarray complete inserts from randomly picked and partially sequenced cDNAs (expressed sequence tags, ESTs) were used (D. Rigola and M. G. M. Aarts, manuscript in preparation). cDNAs, originated from the library made from roots of three-week-old *T. caerulescens* plants (accession La Calamine), raised in hydroponics solution containing half-strength Hoagland's solution and 10 μM $ZnSO_4$ (Assunção *et al.*, 2001). To assess protein and DNA homology with *Arabidopsis thaliana* BLAST (NCBI) database searches were carried out. The current array consists of about 1900 randomly picked cDNAs.

Microarray preparation and fluorescent probes

Amino-modified PCR products of cDNA clones (200 μl) were purified using 96-well multiscreen filter plates (Millipore, Bedford, MA, USA) and suspended in 16 μl spotting solution (3 × SSC supplemented with 1.5 M betaine; SSC: 0.15 M NaCl, 0.015 M Na-citrate, pH 7). These solutions were arrayed from 384-well microtiter plates onto silylated microscope slides (CSS-100 silylated slides; CEL Associates, Houston, Texas, USA) using a DNA array robot (model GMS 417; BioRobotics, Cambridge, UK). The array was printed twice on one microscope slide (2 × 1900 cDNAs). After an incubation time (at least 2 d) the printed arrays were blocked (Huang *et al.*, 2002).

The fluorescent probes were made using the indirect aminoallyl labeling method (http://pga.tigr.org/sop/M004.pdf). Thirty micrograms of total RNA (of four control and four treated samples) were separately reverse-transcribed with SuperScript II (Invitrogen GmbH), then incubated for 2 h in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Biosciences), and finally purified according to the labeling method mentioned above. For each experiment a dye-swap of Cy3-dUTP and Cy5-dUTP was performed.

Hybridization, scanning and hierarchical clustering

Pre-hybridization of slides and necessary steps for hybridization with the Cy3- and Cy5-labeled probes were as described by Huang *et al.*, (2002). Labeled probes were dissolved in 45 μl hybridization buffer and hybridized overnight under a 24 × 50 mm^2 glass cover slip in hybridization chambers (Genemachines, Genetic Solutions, Cambridge, UK). The subsequent washing steps were as described by Huang *et al.*, (2002).

The arrays were scanned using a Fujifilm FLA-8000 scanner (Fuji, Düsseldorf, Germany). Separate images were acquired for each fluorophore at a resolution of 10 μm per pixel. To identify differentially expressed genes the AIDA software (AIDA Image Analyzer 3.51 and AIDA Array Compare 3.51; Raytest, Straubenhardt, Germany) was used. Background fluorescence was calculated as the median fluorescence signal of non-target

pixels around each spot. The obtained ratios were processed by means of freeware. The tool GEPAS (Herrero *et al.*, 2003) is intended to perform tasks like scale transformation, replicate handling, missing value imputation, filtering and normalization of patterns. Suchlike preprocessed data were further processed by the expression profiler software EPCLUST (European Bioinformatics Institute, Cambridge, UK) to perform hierarchical clustering. Presented data show the mean of four separate experiments derived from a homogenized pool of 9 to 11 plants per treatment and accession.

Quantitative RT-PCR

PCRs were performed in a 96-well plate with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany), using SYBR Green to monitor cDNA amplification. 5 μg of total RNA were reverse transcribed using SuperScript II (Invitrogen GmbH) according to the manufacturer's instructions. To remove RNA, RNase H (USB, Cleveland, USA) and RNase A (Promega, Mannheim, Germany) were added and the reaction was incubated at 37 °C for 20 min. cDNA concentration was measured with RiboGreen RNA reagent (Invitrogen GmbH). Primer design was performed with the freeware "Oligo Explorer" of Teemu Kuulasmaa (Kuopio, Finland) for amplicon lengths of between 75 and 160 bp. Primer search was based on the cDNA sequence data. Each PCR contained 12.5 μl of "ABsolute QPCR SYBR® Green ROX Mix" (ABgene, Epsom, UK), 0.2 ng cDNA and 120 nmol of each gene-specific primer in a final volume of 25 μl. The quantitative PCR conditions were: 50 °C (2 min) followed by 95 °C (15 min) for 1 cycle; then 40 cycles at 95 °C (15 s) followed by 60 °C (1 min) were performed. Data were analyzed using ABI Prism SDS Software (v 1.6, Applied Biosystems). The 60 S ribosomal protein L13 [BBC1 protein, 92% similarity to *A. thaliana* (At3g49010)] was used as constitutively expressed control gene. Target gene expression was normalized to the control gene expression by subtracting the average threshold cycle (C_t) value of the 60 S ribosomal protein L13 from the C_t of the target gene for the respective template. Between different cDNAs, the mean C_t for this control gene varied by less than 3.5% (± 0.6 amplification cycles; mean C_t value: 17.5). Quantitative RT-PCR data evaluation is described in "SYBR Green PCR and RT-PCR Reagents" (Applied Biosystems; http://www.appliedbiosystems.com/catalog/). Presented quantitative RT-PCR data are mean values calculated from two technical replicates from one experiment.

Results

Gene-expression dynamics of the hyperaccumulator plant *T. caerulescens* were studied by microarray experiments. The microarray developed for these experiments contained about 1900 ESTs from a cDNA library derived from roots of *T. caerulescens* (accession LC). Hybridization of microarrays was performed with cDNAs of both accessions, Lellingen (LE, nonmetalliferous soil) and La Calamine (LC, metalliferous soil). Highest Zn exposure of LE was limited to 100 μM $ZnSO_4$, because at this level leaf chlorosis developed. LC, however, didn't develop chlorosis until treatment with 1000 μM $ZnSO_4$ (Assunção *et al.*, 2003b). Concentrations of 2 μM $ZnSO_4$ correspond to uncontaminated soils (Weber *et al.*, 2004), while 10 μM $ZnSO_4$ represents a slight zinc stress for nonhyperaccumulator plants. Effects of Zn deficiency on gene expression of LE and LC were determined by no zinc application. After performing hierarchical clustering of the microarray experiments, selections of genes that showed an obvious induction (ratio > 2.0) or repression (ratio < 0.5) in the majority of the experiments remained. Out of this selection functions of some genes are further discussed in more detail.

In Table I *T. caerulescens* accessions LE and LC are compared at the $ZnSO_4$-concentrations mentioned above. Many, but not all of the ESTs could be aligned to sequences provided by diverse databases. Some unknown genes showed a very interesting transcriptional profiling, and an example is given in Tables I and III. Glycoprotein EP1 of the accession LC was clearly induced in all tested $ZnSO_4$-concentrations, compared to LE (Table I). In carrot the expression of this gene was detected in cells that are located in the epidermis of the root and in the root cap. A possible involvement of EP1 in cell elongation is suggested (Van Engelen *et al.*, 1993). Also ubiquitin extension protein 1 was expressed clearly stronger throughout in LC than in LE. This protein is responsible for covalent addition of polyubiquitin to other proteins, targeting the tagged protein for destruction or different fates (Larson and Wang, 2002). Com-

Table I. Selected gene ratios in roots expressed at high or low levels in microarray experiments comparing the two *T. caerulescens* accessions Lellingen (LE) and La Calamine (LC). 0, 2, 10, 100 indicate the $ZnSO_4$-concentrations (0, 2, 10, 100 μM $ZnSO_4$) that were used for plant treatments. Values (induction or repression) refer to the italic typed accession. Asterisk symbols (*) indicate that the same gene is also described in Table III. SD means standard deviation. Repression: ≤ 0.5; induction: ≥ 2.0.

Gene annotation	Similarity to *A. thaliana*[a]	LE0– *LC0*	SD	LE2– *LC2*	SD	LE10– *LC10*	SD	LE100– *LC100*	SD
No information*		20.5	4.1	2.1	0.0	4.9	1.3	4.4	1.2
Glycoprotein EP1	76% to At1g78850	2.6	0.5	2.9	0.5	4.2	1.3	3.4	1.0
Ubiquitin extension protein 1	76% to At3g52590	1.7	0.3	4.2	1.3	2.9	0.5	2.1	0.1
Class 1 non-symbiotic hemoglobin*	85% to At2g16060	2.2	0.6	4.7	0.8	1.2	0.2	0.5	0.1
Cytochrome p450 monooxygenase	no *A.t.* [*Zea mays*]	0.4	0.2	0.3	0.2	0.4	0.1	0.1	0.1
Glutamine synthetase*	87% to At1g66200	0.4	0.2	0.1	0.0	0.3	0.2	0.4	0.3
Senescence associated protein	no *A.t.* [*Pisum sat.*]	0.5	0.3	0.4	0.3	0.4	0.1	0.1	0.1
Fructose bisphosphate aldolase*	93% to At3g52930	0.7	0.1	0.1	0.1	0.6	0.2	0.5	0.3
Phenylalanine ammonia-lyase*	96% to At2g37040	0.7	0.1	0.2	0.0	0.7	0.2	0.5	0.1

[a] AGI code; values are average ratios of normalized signals of Lellingen versus La Calamine, which were calculated using AIDA software from four separate experiments derived from a homogenized pool of 9–11 plants per treatment and accession.

pared to LE, the class 1 non-symbiotic hemoglobin gene of LC was induced at absolute Zn deficiency (0 μM) and at 2 μM $ZnSO_4$ in soil (Table I). While expression of the gene was similar at 10 μM $ZnSO_4$ for both accessions, it was repressed in LC at 100 μM $ZnSO_4$. It is active in germinating seedlings and can be induced by hypoxia and increased sucrose supply (Hunt *et al.*, 2001). Furthermore it could play a role by maintaining the energy status of cells that have ATP demands that are not readily met by mitochondrial respiration (Hill, 1998). Compared to LE transcription of a cytochrome p450 monooxygenase, of glutamine synthetase and of a senescence-associated protein was clearly repressed in LC at every tested Zn concentration (Table I). The heme-containing enzymes of the cytochrome p450 monooxygenase family mostly catalyze NADPH- and O_2-dependent hydroxylation reactions (Chapple, 1998). Glutamine synthetase is responsible for the initial assimilation of ammonia into organic compounds. According to the comparison between the two accessions a repression of fructose bisphosphate aldolase, an enzyme of the glycolysis pathway, and phenylalanine ammonia-lyase (PAL) was found at concentrations of 2 and 100 μM $ZnSO_4$ (Table I). Otherwise a down-regulation of the two transcripts could be observed in LC. PAL is a key-enzyme of the phenylpropanoid biosynthesis pathway and is involved in the low-level accumulation of phenolics in most cell types. Moreover it also responds to various environmental stimuli such as wounding, light, nutrient supply and plant hormones (Hahlbrock and Scheel, 1989).

Expression ratios of some selected candidate genes were confirmed by quantitative RT-PCR. Table II shows the ratios of three genes determined by microarray experiments and by qRT-PCR. Induction of glycoprotein EP1 and ubiquitin extension protein 1 in LC was thus verified. Ratios identified by qRT-PCR were higher than the array ratios. Differences in gene expression data found by microarray technologies or by qRT-PCR are well-known in the literature (Holland, 2002; Czechowski *et al.*, 2004). In the case of class 1 non-symbiotic hemoglobin confirmation of induction in LC failed at a concentration of 0 μM $ZnSO_4$. Otherwise induction respectively repression referred to LC corresponded to the array results.

In Table III effects of the different $ZnSO_4$-concentrations on the expression of selected genes either in LE or in LC are given. Except for PAL, the transcription of glutamine synthetase, fructose bisphosphate aldolase and a putative aquaporin gene were down-regulated in LE at 0 μM $ZnSO_4$ compared to 2 μM $ZnSO_4$. However, when 100 μM was compared to 2 μM $ZnSO_4$, an induction of all four genes was visible in LE (100 μM). A clear induction of these genes was also found in LC at absolute Zn deficiency (0 to 2 μM), but also at high Zn concentrations (100 to 2 μM and 1000 to 10 μM $ZnSO_4$). While the expression of methionine synthase was clearly down-regulated in LE at Zn deficiency, an induction was visible for *S*-adenosyl-

Table II. Comparison of the expressions of three selected genes (from Table I) found in microarray experiments with expressions obtained by quantitative RT-PCR analysis. Ratios referring to roots were calculated by comparing the signals of the two *T. caerulescens* accessions LE and LC at different $ZnSO_4$-concentrations. Other information on this Table can be found in the caption of Table I.

Gene annotation	LE0–*LC0*	LE2–*LC2*	LE10–*LC10*	LE100–*LC100*	
Glycoprotein EP1	2.6	2.9	4.2	3.4	array
	17.9	36.0	9.3	7.9	qRT-PCR
	(16.0–19.9)	(30.2–42.9)	(8.3–10.5)	(7.5–8.3)	
Ubiquitin extension protein 1	1.7	4.2	2.9	2.1	array
	3.8	9.2	6.9	6.5	qRT-PCR
	(3.5–4.1)	(8.2–10.3)	(6.3–7.6)	(5.5–7.6)	
Class 1 non-symbiotic hemoglobin*	2.2	4.7	1.2	0.5	array
	0.9	4.3	1.2	0.4	qRT-PCR
	(0.9–1.0)	(3.8–4.9)	(1.1–1.3)	(0.4–0.4)	

qRT-PCR values are mean values calculated from two technical replicates from one experiment. Target gene expression was normalized to the control gene expression by subtracting the C_t of the 60 S ribosomal protein L13 (BBC1) from the C_t of the target gene for the respective template. For a detailed description of qRT-PCR data evaluation see "Materials and Methods".

methionine synthase and for methionine synthase at 100 μM $ZnSO_4$, compared to 2 μM $ZnSO_4$. In LC however, no difference in expression was found for methionine synthase in contrast to S-adenosylmethionine synthase, when 2 and 0 μM $ZnSO_4$ were compared. Expression of both enzymes was up regulated in LC treated with increased $ZnSO_4$-concentrations. Methionine synthase catalyzes the formation of methionine and *S*-adenosylmethionine synthase is responsible for the formation of *S*-adenosylmethionine. In LE a class 1 non-symbiotic hemoglobin and an alcohol dehydrogenase were induced at Zn deficiency and at 100 μM $ZnSO_4$-concentration, each compared to 2 μM $ZnSO_4$ (Table III). However, in LC little difference in expression was found at Zn deficiency for these two genes. Furthermore, supply of 100 and 1000 μM $ZnSO_4$, compared either to 2 or to 10 μM $ZnSO_4$, resulted in a down-regulation of these genes. Alcohol dehydrogenase is sometimes related to hypoxic/anoxic conditions in plants (Peng *et al.*, 2001). In LE a putative zinc transport protein was induced, when zinc wasn't supplied, whereas it was repressed at high Zn supply (Table III). In LC, a down-regulation of this zinc transporter was identified only at a concentration of 1000 μM $ZnSO_4$. Otherwise there was no significant difference in expression at Zn deficiency and at 100 μM $ZnSO_4$, compared to 2 μM $ZnSO_4$.

As described above quantitative RT-PCR was performed in order to verify the array results. Table IV shows the ratios of two genes determined by microarray experiments and by qRT-PCR. In LE an induction of alcohol dehydrogenase could be demonstrated in both treatments. In LC repression caused by a supply of 100 and 1000 μM $ZnSO_4$ became more apparent when the ratios were determined by qRT-PCR. An up-regulation of class 1 non-symbiotic hemoglobin in LE for both treatments was verified. The repression of this gene in LC found out by array data for the three treatments was in accordance with the results of the qRT-PCR.

Discussion

Microarray technology is an important and powerful tool for transcript profiling of any species. Results of microarray hybridizations can be verified by RNA gel blot analysis or quantitative RT-PCR.

Performing hierarchical clustering of the microarray results revealed some genes with unknown functions that showed strong induction or repression in particular Zn treatments. This is of special interest, because these genes are metal-responsive and maybe a unique feature of the hyperaccumulator plant *T. caerulescens.*

Next to genes of unknown function mostly genes with a putative function were found to be differentially expressed. A glycoprotein EP1 gene was basically stronger induced in the accession LC that is adapted to the metalliferous soil. This finding is in accordance with an induction of a putative

Table III. Selected gene ratios in roots expressed at high or low levels in microarray experiments comparing different $ZnSO_4$-treatments of plants of the *T. caerulescens* accession LE or LC. 0, 2, 10, 100, 1000 indicate the used $ZnSO_4$-concentrations (0, 2, 10, etc. μM $ZnSO_4$). Values (induction or repression) refer to the italic typed $ZnSO_4$-concentration. Asterisk symbols (*) indicate that the same gene is also described in Table I. SD means standard deviation. Repression: ≤ 0.5; induction: ≥ 2.0.

Gene annotation	Similarity to *A. thaliana*[a]	LE2– *LE0*	SD	LE2– *LE100*	SD	LC2– *LC0*	SD	LC2– *LC100*	SD	LC10– *LC1000*	SD
Glutamine synthetase*	87% to At1g66200	0.5	0.1	2.3	1.2	7.0	0.8	4.2	2.7	6.8	2.8
Fructose bisphosphate aldolase*	93% to At3g52930	0.5	0.2	1.9	0.8	3.3	0.4	2.5	1.3	3.0	0.7
Putative aquaporin	84% to At2g36830	0.4	0.1	1.8	0.5	6.3	0.5	2.1	0.5	6.2	1.6
Phenylalanine ammonia-lyase*	96% to At2g37040	0.8	0.2	1.9	0.2	2.1	0.3	2.6	0.1	2.1	0.5
S-Adenosylmethionine synthase	86% to At4g01850	0.7	0.2	2.3	0.2	2.9	0.8	3.8	1.7	3.4	1.0
Methionine synthase	89% to At3g03780	0.4	0.1	1.9	0.5	0.9	0.2	2.6	0.8	5.0	1.0
Alcohol dehydrogenase	69% to At1g77120	2.0	0.2	2.9	0.2	1.0	0.2	0.8	0.2	0.5	0.1
Class 1 non-symbiotic hemoglobin*	85% to At2g16060	1.9	0.3	2.3	0.4	0.6	0.1	0.3	0.1	0.3	0.1
Putative zinc transport protein	88% to At1g05300	1.8	0.2	0.2	0.0	1.3	0.2	0.8	0.2	0.4	0.0
No information*		0.4	0.0	0.6	0.2	3.3	0.8	0.7	0.2	0.0	0.0

[a] AGI code; values are average ratios of normalized signals of one $ZnSO_4$-concentration versus another $ZnSO_4$-concentration, which were calculated using AIDA software from four separate experiments derived from a homogenized pool of 9–11 plants per treatment and accession.

EP1-like glycoprotein in roots of *Arabidopsis halleri*, collected at a metal-contaminated site, compared to normal-grown *A. thaliana* plants (Weber *et al.*, 2004). As glycoprotein EP1 expression is mainly located in the epidermis of the root and in root caps (Van Engelen *et al.*, 1993), this glycoprotein could play a role in adaptation to metalliferous soil.

Class 1 non-symbiotic hemoglobins are expressed in mature roots and according to Taylor *et al.*, (1994) mainly in root tissue under anaerobic conditions. In addition these genes may be important in maintaining the energy status of cells (Hill, 1998). Comparing the two accessions the highest induction of this gene was found in LC at a treatment of 2 μM $ZnSO_4$. Considering this treatment, LE reacted to absolute Zn deficiency and to an increased Zn level with an up-regulation of the class 1 non-symbiotic hemoglobin. In LC, however, this gene was always down-regulated, comparing 0, 100 and 1000 μM $ZnSO_4$ to 2 respectively 10 μM $ZnSO_4$. Consequently, the roots of LE could suffer from oxygen stress or from ATP deficiency when both 0 and 100 μM $ZnSO_4$ were supplied, compared to 2 μM $ZnSO_4$, that corresponds to a normal soil zinc concentration (0.8 μM Zn^{2+}). This normal Zn supply however obviously caused a high expression of the class 1 non-symbiotic hemoglobin in the heavy metal adapted roots of LC, compared to LE, whereas no or increased Zn concentrations seem to extenuate a possible oxygen stress or a possible ATP deficiency in LC. These observations indicate an involvement of the class 1 non-symbiotic hemoglobin in the elevated tolerance of LC roots against contaminated soil types, although connections between Zn treatments and anaerobic conditions or higher ATP demand have to be elucidated. However, expression of alcohol dehydrogenase, a known anaerobic response gene (Peng *et al.*, 2001), was similar to the expression of the class 1 non-symbiotic hemoglobin (see Table III). In fact the *Thlaspi* plants were grown up in hydroponic solutions and might have suffered from anaerobiosis. As all plants were grown under the same conditions, a link between Zn supply and hypoxic conditions may be indicated.

Weber *et al.* (2004) and Becher *et al.* (2004) discuss nicotianamine synthase as a key factor for metal-hyperaccumulation. Regrettably this enzyme was not included on our microarray. But still, after the process of hierarchichal clustering, induction of enzymes like methionine synthase and *S*-

Table IV. Comparison of the expressions of two selected genes (from Table III) found in microarray experiments with expressions obtained by quantitative RT-PCR analysis. Ratios referring to roots were calculated by comparing the signals of different $ZnSO_4$-treatments of plants of the accession LE or LC. Other information on this Table can be found in the caption of Table III.

Gene annotation	LE2–*LE0*	LE2–*LE100*	LC2–*LC0*	LC2–*LC100*	LC10–*LC1000*	
Alcohol dehydrogenase	2.0	2.9	1.0	0.8	0.5	array
	2.5	2.2	0.7	0.4	0.4	qRT-PCR
	(2.2–2.8)	(1.9–2.5)	(0.7–0.7)	(0.3–0.4)	(0.3–0.4)	
Class 1 non-symbiotic hemoglobin*	1.9	2.3	0.6	0.3	0.3	array
	1.9	1.8	0.4	0.2	0.3	qRT-PCR
	(1.7–2.2)	(1.6–2.0)	(0.4–0.5)	(0.2–0.2)	(0.2–0.3)	

qRT-PCR values are mean values calculated from two technical replicates from one experiment. For additional information see Table II.

adenosylmethionine synthase that are involved in the biosynthetic pathway of nicotianamine were found. Thus, methionine is activated to *S*-adenosylmethionine (SAM) by SAM synthetase as part of the Yang cycle, and three molecules of SAM are combined to form nicotianamine by nicotianamine synthase (Negishi *et al.*, 2002). Induction of these genes was mainly visible in LC at increased Zn concentrations. In LE an up-regulation was only found at 100 μM $ZnSO_4$, compared to 2 μM $ZnSO_4$. Ethylene can also be synthesized from SAM. Ethylene is a plant hormone responsible for senescence and fruit ripening amongst others. Although SAM synthetase was induced at increased Zn concentrations mainly in LC, the expression of a senescence associated protein and of glutamine synthetase was down-regulated in LC, compared to LE at every Zn concentration. According to Miflin and Habash (2002) glutamine synthetase may also accelerate leaf development. Compared to LE, phenylalanine ammonia-lyase, an enzyme which responds to many environmental stresses (Hahlbrock and Scheel, 1989), was down-regulated in LC at all four Zn concentrations. In addition it was induced in LC at absolute Zn deficiency and at increased Zn concentrations. These observations stress out that LC roots could deal with heavy metal stress in a better way, compared to LE roots.

In LE Zn uptake seems to be supported by an up-regulation of a putative zinc transport protein, when no zinc is supplied. However, at 100 μM $ZnSO_4$ this zinc transporter gene was down-regulated, indicating a repression of Zn uptake. In LC repression of this gene was only evident at 1000 μM $ZnSO_4$, compared to 10 μM $ZnSO_4$. This fits very well with the reduced tolerance of LE for high Zn concentrations as found by Assunção *et al.* (2003b).

In conclusion comparative transcript profiling of *T. caerulescens* plants from metalliferous and non-metalliferous soil has identified putative genes that are affected by plant heavy metal tolerance, accumulation and transport. Additionally array data of selected genes were verified by qRT-PCR. Genes described in this paper only mirror a narrow selection of putative genes involved in Zn hyperaccumulation.

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Assunção A. G. L., Da Costa Martins P., De Folter S., Vooijs R., Schat H., and Aarts M. G. M. (2001), Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ. **24**, 217–226.

Assunção A. G. L., Schat H., and Aarts M. G. M. (2003a), *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytol. **159**, 351–360.

Assunção A. G. L., Bookum W. H., Nelissen H. J. M., Voojis R., Schat H., and Ernst W. H. O. (2003b), Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. New Phytol. **159**, 411–419.

Becher M., Talke I. N., Krall L., and Krämer U. (2004), Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J. **37**, 251–268.

Brown S. L., Chaney R. L., Angle J. S., and Baker A. J. M. (1995), Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. Soil Sci. Soc. Amer. J. **59**, 125–133.

Chapple C. (1998), Molecular-genetic analysis of plant cytochrome p450-dependent monooxygenases. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**, 311–343.

Cunningham S. D. and Berti W. R. (1993), Remediation of contaminated soils with green plants: an overview. In Vitro Cell Biol. **29**, 207–212.

Czechowski T., Bari R. P., Stitt M., Scheible W.-R., and Udvardi M. K. (2004), Real-time RT-PCR profiling over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J. **38**, 366–379.

Hahlbrock K. and Scheel D. (1989), Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 347–369.

Herrero J., Al-Shahrour F., Díaz-Uriarte R., Mateos Á., Vaquerizas J. M., Santoyo J., and Dopazo J. (2003), GEPAS, a web-based resource for microarray gene expression data analysis. Nucl. Acids Res. **31**, 3461–3467.

Hill R. D. (1998), What are haemoglobins doing in plants? Can. J. Bot. **76**, 707–712.

Holland M. J. (2002), Transcript abundance in yeast varies over six orders of magnitude. J. Biol. Chem. **277**, 14363–14366.

Huang X., Rad v. U., and Durner J. (2002), Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells. Planta **215**, 914–923.

Hunt P. W., Watts R. A., Trevaskis B., Llewelyn D. J., Burnell J., Dennis E. S., and Peacock W. J. (2001), Expression and evolution of functionally distinct haemoglobin genes in plants. Plant Mol. Biol. **47**, 677–692.

Larson C. N. and Wang H. (2002), The ubiquitin superfamily: members, features, and phylogenies. J. Prot. Res. **1**, 411–419.

Lombi E., Zhao F. J., Dunham S. J., and McGrath S. P. (2000), Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. New Phytol. **145**, 11–20.

Meerts P. and Van Isacker N. (1997), Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. Plant Ecol. **133**, 221–231.

Miflin B. J. and Habash D. Z. (2002), The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J. Exp. Bot. **53**, 979–987.

Negishi T., Nakanishi H., Yazaki J., Kishimoto N., Fujii F., Shimbo K., Yamamoto K., Sakata K., Sasaki T., Kikuchi S., Mori S., and Nishizawa N. K. (2002), cDNA microarray analysis of gene expression during Fe-deficiency stress in barley suggests that polar transport of vesicles is implicated in phytosiderophore secretion in Fe-deficient barley roots. Plant J. **30**, 83–94.

Peng H.-P., Chan C.-S., Shih M.-C., and Yang S. F. (2001), Signaling events in the hypoxic induction of alcohol dehydrogenase gene in *Arabidopsis*. Plant Physiol. **126**, 742–749.

Reeves R. and Baker A. (2000), Metal accumulating plants. In: Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment (Raskin I. and Ensley B., eds.). John Wiley & Sons, New York, USA, pp. 193–229.

Schat H., Llugany M., and Bernhard R. (2000), Metal-specific patterns of tolerance, uptake, and transport of heavy metals in hyperaccumulating and non-hyperaccumulating metallophytes. In: Phytoremediation of Contaminated Soils and Water (Terry N. and Banuelos G., eds.). CRC Press LLC, Boca Raton, FL, USA, pp. 171–188.

Taylor E. R., Nie X. Z., MacGregor A. W., and Hill R. D. (1994), A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. Plant Mol. Biol. **24**, 853–862.

Van Engelen F. A., Hartog M. V., Thomas T. L., Taylor B., Sturm A., Van Kammen A., and De Vries S. C. (1993), The carrot secreted glycoprotein gene EP1 is expressed in the epidermis and has sequence homology to *Brassica* S-locus glycoproteins. Plant J. **4**, 855–862.

Vassilev A., Schwitzguébel J.-P., Thewys T., v. d. Lelie D., and Vangronsveld J. (2004), The use of plants for remediation of metal-contaminated soils. Sci.World J. **4**, 9–34.

Weber M., Harada E., Vess C., v. Roepenak-Lahaye E., and Clemens S. (2004), Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulator factors. Plant J. **37**, 269–281.